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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requesitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Prof. Dr. Ricardo Jorge Guerra Calado, Investigador Auxiliar, CESAM - Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro

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Um dia acreditei que conquistas como essa me seriam possíveis, mas sempre soube que elas só se tornariam reais se acreditasse com a alma. À Deus agradeço por todo carinho e cuidado em preparar meu caminho até aqui; ao meu avô Amaro e avó Erondina agradeço por me enxergarem melhor do que sou de fato e por acreditarem desde o princípio que essa vitória seria possível. Aos meus pais agradeço em especial pelos valores ensinados, dentre eles o que com perseverança e honestidade nossos objetivos podem ser alcançados com sucesso. Aos amigos e familiares, de longe e de perto, agradeço por todo suporte e compreensão nos momentos fáceis e difíceis, tornando minha jornada mais leve e finalmente possível. Acho que só quem completa uma etapa como essa é capaz de entender que nossos agradecimentos deveriam ser maiores que a própria dissertação. Muitos foram os Mestres e colegas de profissão que também marcaram meu caminho e a eles gostariade agradecer por dividirem essa paixão comigo. À todos, com o coração repleto de gratidão, confesso: essa conquista seria impossível sem vocês!

Palavras-chave Pesca com cianeto, Tiocianato, Peixes marinhos, Recifes de coral

Resumo

A pesca com cianeto $(CN-)$ é uma técnica destrutiva utilizada na colheita de peixes vivos de recifes de coral. Estes organismos apresentam elevado valor económico e são destinados tanto para o consumo humano como para o abastecimento da indústria mundial de aquários marinhos. Diversas são as técnicas capazes de detectar a presença do cianeto $(CN-)$ em peixes, contudo ainda não há um consenso entre a comunidade científica e os comerciantes sobre qual destas técnicas será a mais eficaz, uma vez que as mais utilizadas ainda são de caráter invasivo. Neste trabalho foi utilizada uma técnica não invasivo e não destrutiva, e mais eficiente, no que diz respeito ao tempo de análise, onde através do uso da fibra óptica (FO) podem ser detectados peixes contaminados com cianeto num tempo médio $<$ 6 min. por meio da excreção de tiocianato $(SCN-)$. Produto de excreção do $(CN-)$, esse metabolito permite a desintoxicação dos peixes marinhos expostos ao contaminante pelas vias urinárias e os níveis anormais de SCN- presentes na água marinha indicarão se os exemplares foram ou não expostos ao envenenamento por $CN-$. A metodologia (FO) foi capaz de detectar níveis ainda que residuais de $SCN(> 3,16mgL-1)$ na água marinha e os níveis base para os organismos não contaminados foram utilizados como referência para classificação de presença ou ausência de contaminação. Nesse estudo exemplares de Amphiprion clarkii cultivados em cativeiro foram expostos a um pulso de solução de $CN-$ durante 60 s para as concentrações de $12,5e25,0mgL-1$ e os resultados obtidos para o $CN-$ excretado, pós-exposição ao longo de 28 dias, foram de até $6,96 \pm 0,03$ e $9,84 \pm 0,03mgL-1$ de $SCN-$ (respectivamente). Apesar da necessidade de mais investigação para diminuir a ocorrência de falsos negativos e positivos, a metodologia testada permite uma rápida detecção do $SCN-$ sem o sacrifício dos espécimes analisados.

Keywords Cyanide fishing, Thiocyanate, Marine fish, Coral Reef

Abstract Cyanide fishing is a method employed to collect live marine fish on coral reefs. They are either shipped to markets for human consumption in Southeast Asia or supplied to the marine aquarium trade worldwide. Although several techniques can be used to detect cyanide in reef fish, there is still no testing method which can be used to survey the whole supply chain. Most methods for cyanide detection are time consuming and require the sacrifice of surveyed fish. Thiocyanate anion $(SCN-)$ is a metabolite produced by the main metabolic pathway for cyanide anion $(CN-)$ detoxification. Our study employed an optical fiber (OF) methodology $(analyticaltime < 6min)$ to detect $SCN-$ in a non-invasive and non-destructive manner. Our OF methodology is able to detect trace levels $(> 3.16\mu gL - 1)$ of this compound in seawater. Given that marine fish exposed to cyanide excrete $SCN-$ in the urine, abnormal levels of SCN- present in the seawater holding live reef fish can indicate that the surveyed specimens were most likely exposed to cyanide. In our study, captive-bred clownfish (Amphiprion clarkii) pulse exposed during 60 s to either 12.5 or $25mgL - 1$ of $CN-$ excreted up to 6.96 ± 0.03 and $9.84 \pm 0.03\mu gL - 1$ of $SCN-$(respectively) during 28 days following exposure. No detectable levels of $SCN-$ were recorded in the water holding control organisms (not exposed to CN) or in batches of prepared synthetic seawater. While further research is necessary, our methodology can allow the rapid detection of the presence of $SCN-$ which indicates that live reef fish were collected with cyanide.

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Chapter 2

Presents our journal containing the developed work and all the obtained results, concluding that the OF cyanide detection methodology is easier, faster, safer and cheaper to use than other equivalent techniques available.

Chapter 3

Presents the final considerations and future steps containing suggestions to increase the performance of the OF sensor and the methodology application.

Annex – 1

Presents a PDF file of the manuscript describing in detail the methodology employed in this work to determine thiocyanate (SCN-) in seawater.

Chapter 1

State of the Art

Chapter 1

State of the Art

1. Habitat destruction

Coral reefs are probably the most biologically diverse ecosystem on Earth and have been a source of fascination for their myriad of life forms and colours since their discovery. A single reef may be the home of over 200 species of coral, 300 species of fish and between 10.000 and 100.000 invertebrates [1]. As one of the most productive ecosystems, it provides food to millions of people in coastal areas around the world, since a coral reef of about one km^2 may provide food for 2.500 human beings per year, provided that it is healthy and intact [1, 2]. This ecosystem is also an important source of animal protein and income to local population (often living at subsistence levels), playing an important role from a biodiversity point of view, as well as for scientific and educational purposes. Furthermore, coral reefs act as a natural protection against wave erosion, as a waste treatment area (by nitrogen fixation), as a nursery, a habitat for resident and transient populations, a feeding place and a food production area, a source of primary production and extractable materials (fish, seaweed, materials for medicine, jewellery, coral blocks, sand, etc), and opportunity for recreational activities such as commercial and non-commercial use (fishing, recreation and cultural-religious values). These areas are responsible to maintain the ecological equilibrium between the shoreline to the open ocean [1, 3-5], as well as to contribute to the development of local income generation, beneficial to local economies and small markets [5].

However, this ecosystem is being degraded at an alarming rate in all tropical oceans, posing a threat to food security, an issue which will particularly affect the poorest areas [6]. The attention to the socioeconomic dimension of this problem also stems from the fact that humans are responsible for much of the decline in coral reef health, for instance, through marine pollution, coral mining, sedimentation and destructive fishing practices [5, 7].

Throughout the Indo-Pacific Ocean, only a small fraction of all coral reefs are in pristine state and these exist in remote areas far from human settlements. Many reefs are seriously degraded already, perhaps as many as 80% to 90% in countries like the Philippines and Indonesia, where most of the destruction comes from over-exploitation and the use of destructive fishing techniques [2, 5, 7]. Following this trend, cyanide fishing is an excellent example of a destructive fishing technique, since it is estimated that over 500 metric tons of cyanide may be used annually to collect live fish on Philippine reefs [7].

Studies related to the cyanide environmental effects have underlined coral bleaching as a most common consequence of chemical abuse of coral reefs areas [1, 5, 7]. Aiming to explain how this process occurs, the next section will present a brief resume of coral bleaching causes and their consequences on the marine ecosystem.

1.1.1. Coral bleaching

Reefs are rather productive shallow water marine ecosystems that are based on rigid lime skeletons formed through successive growth, deposition and consolidation of remains of reef building corals and coralline alga e [8]. The basic units of reef growth are the coral polyps and the associated symbiotic algae that lives in coral tissues - the zooxanthellae. This symbiotic relationship allows zooxanthellae to be protected and survive inside another organism and, by photosynthesis, provide food to their host (corals, anemone, etc); this mutualism is the key factor explaining both the productivity of reefs and the rather strict environmental requirement of corals [1, 5].

The mechanism of coral bleaching has been described as the loss of symbiotic single-celled algae (zooxanthellae) that normally colonize the tissues of corals and suddenly is expulsed by the coral polyps and can occur by different forms of stress (including unusual increase of temperature, oxygen deficiency, presence of toxic pollutants in the water, increase of ultraviolet radiations possibly due to ozone depletion, the activity of secondary pathogens following physical stress, or the combination of all the aforementioned factors) [4, 8]. After the expulsion, if the zooxanthellae do not find another host, or if the host loses all its symbiotic partners, they will die and the bleaching process is initiated [4].

The bleaching of corals was first recorded on Guam in 1962 and the main cause was related to the anthropogenic influence of washing out of the soil, as a consequence of military construction activity [4]. Later, several episodes related to widespread bleaching and death of corals, during 1970's and 1980's, were described as a consequence of epizootic disease along with some natural environmental changes, such as the effects of El Nino, hurricanes, increased sedimentation and temperature, and changes in nutrient concentrations. [1, 4, 8, 9]. According to Sorokin (1995), coral reef deterioration in the Atlantic and Pacific Oceans may also been caused by human activities. This anthropogenic stress is recognized as significantly more dangerous than other abiotic stressors; as in most cases, it is not only an isolated event but can rather be a permanent stress source with the tendency towards an increase with time. In conditions of cumulative impact of physical and anthropogenic stress, the inhibition and destruction of reef systems can rapidly become irreversible.

Thus, the anthropogenic impacts, as a consequence of unlimited fisheries, industrial and recreational human activities, have become a massive anthropogenic stress since Second World War. The construction of numerous military bases, ports and channels began, along with the collection of shells and corals by the militaries and their families, rapidly set up a market of souvenirs. After the War, the main goal of the governments in developing costal and island states was to provide food and shelter to the population; following this idea, the stimulation of the super-exploitation of reef resources by various activities (such as intensive fishery, extraction of lime and sand for the construction industry and the collection of souvenirs for international trade) became the factor of danger for the reef existence. [4, 6, 10]. Destruction was aggravated by the development of diving equipments, boat motors and new fishing techniques (i.e. high quality nets, radio/satellites fish finders and fishing chemicals, such as cyanide) [1, 4, 5, 11]. Ranking destructive fishing techniques in countries like Indonesia and Philippines, cyanide fishing is probably only second to blast fishing [7].

Coral bleaching caused by cyanide promotes the disruption in the zooxanthellae photosynthesis process and results in death of coral polyps [1]. Cyanide fishing kills targeted and non-targeted specimens [7]. However, CESAR (2000) also assumed that the cyanide toxicity to corals under experimental conditions in itself does not prove for the degradation on the scale of a reef. This is because the rate of coral loss due to cyanide fishing may be lower than the rate of natural coral growth, or even because cyanide is dissipated too rapidly by water currents to affect exposed corals under natural conditions. It is also unclear how reef degradation from cyanide fishery for food compares to the problem of reef degradation caused by other destructive fishing techniques [1]. If cyanide fishing isn't the main responsible cause for the bleaching process, it is well know that cyanide is a highly toxic chemical that can act under different forms in the reef ecosystem and most certainly contributes to its degradation [1, 7].

2. Cyanide

2.1.1. Chemical definition and coordination chemistry

According to the IUPAC gold book, cyanides are classified as Salts and C-organyl derivatives of hydrogen cyanide, HC≡N, e.g. CH3C≡N methyl cyanide (acetonitrile); NaCN sodium cyanide; PhC(=O)CN benzoyl cyanide; KCN potassium cyanide, and others [12].

NaCN and KCN are classified as ionic-cyanides and are the chemicals most commonly employed in cyanide fishing in coral reefs [7] . Those salts are mostly prepared by the direct reaction of hydrogen cyanide with the respective alkali in closed systems. Sodium cyanide is also prepared to a lesser extent by melting sodium chloride with calcium cyanamide or by heating sodium amide salt with carbon [13-15].

2.1.2. Sodium cyanide (NaCN)

NaCN is a hygroscopic crystalline powder with a faint bitter almond-like odour. Common synonyms are cyanide sodium and hydrocyanide acid-sodium. Commercially available NaCN generally achieves a purity of 95%-98%. Soluble in water, slight soluble in alcohol, the aqueous solution of this chemical is strongly alkaline and rapidly decomposes. NaCN produces hydrogen cyanide on contact with acids or acids salts [13-15].

2.1.3. Potassium cyanide (KCN)

KCN is a white deliquescent solid with an odour of hydrogen cyanide. Common synonyms are hydrocyanic acid, potassium salt and cyanide potassium. KNC is commercially available at 95% purity. Soluble in water, slight soluble in alcohol, the aqueous solution of KCN in water is strongly alkaline and in contact with acids or acid salts, produce hydrogen cyanide [13-15].

2.1.4. Occurrence and applications

The principal natural sources of cyanide are over 2000 plant species, including vegetables and fruits that contain cyanogenic glycosides, which can release cyanide on hydrolysis when ingested. Acting with bacteria and fungi can also release hydrogen cyanide into the atmosphere from natural biogenic process [2][3][15].
Cyanides comprise a wide range of compounds, of varying degrees of chemical complexity. They can affect the environment in gaseous, liquid or solid forms, from a broad range of natural and anthropogenic activities, and have been used as medical compounds for pest control and the organic chemistry industry. Cyanides have the potential to be transported over long distances from their emission sources. However, even if not biocumulative into the ecosystem, those elements present high toxicity with the cyanide anion CN^- , usually the primary toxic agent, regardless of origin [15]. NaCN and KCN, specifically, are used in many processes, such as at the recovery steps of gold and silver, metal degreasing and separation, photography process, base metal flotation, and also as a strong anaesthetic to capture reef fish. The whole process involving the cyanide fishing, the chemical depuration, and they effects as well, will be present in the next section.

3. Cyanide fishing

For a long time reef fisheries have provided food for people from tropical coastal areas; however, since the birth of the aquarium industry it has become the main destination of live reef fish [4]. Aiming to satiate this multimillion dollar business, the capture of these organisms became common in the Indo-Pacific Ocean, and about 20 000 hundred thousand aquarium live fish were exported annually from Philippines alone during the 1990's. [4]. Cyanide fishing is a fast method to stun and collect reef fish in general-and, since the early 1960's, sodium cyanide has been used in the Philippines [16]. This practice rapidly spread throughout southeast Asia, in association with the live fish trades, to collect tropical marine fish for the marine aquarium industry, with live reef fish being exported worldwide; it is important to highlight that some fish species (mostly groupers) are also shipped alive to supply high end consumers in Hong Kong and China [7]. According to Colette (2003), the Philippines, Indonesia, the Solomon Islands, Sri Lanka, Australia, Fiji, the Maldives and Palau supplied more than 98% the total number of fish exported between the years 1997 and 2002. GMAD also showed that during the same period, countries such as the United States, the United Kingdom, the Netherlands, France and Germany were the main importing countries of destination, comprising 99% of all marine ornamental fish imports. In the same report, Pomacentridae was the most representative family composing 43% of all trade family analyzed, followed by Acanthuridae, Labridae, Gobiidae, Chaetodontidae, Callionymidae, Microdesmidae, Serranidae and Blenniidae [17]. Table 1 presents a summary of the total number of marine ornamental fish, represented by most commonly traded families, exporters between 1997 and 2002 in GMAD adapted from Colette (2003).

Table 1- Main source countries of exporter's marine ornamental fish adapted from [17].

Origin Country	No. of fish exported (exporters' data)	% of total no. of fish traded
Philippines	1,523,854	43
Indonesia	943,059	26
Solomon Islands	416,262	12
Sri Lanka	183,537	5
Australia	173,323	5
Fiji	131,746	4
Maldives	78,018	2
Palau	63,482	2
Total	3,513,281	99

Table 2 presents a summary of the total number of marine ornamental fish imported, represented by most commonly traded families and the main importing countries, between 1997 and 2002 in GMAD adapted from Colette (2003).

Table 2 - Main source countries of importer's marine ornamental fish adapted from [17].

Destination Country	No. of fish imported (exporters' data)	% of total no. of fish traded
USA	1,462,347	41
Unknown	788,230	22
Taiwan	244,454	7
Japan	223,613	6
Hong Kong	152,738	4
France	132,439	4
Germany	119,739	3
Netherlands	117,248	3
Italy	70,686	2
United Kingdom	48,911	1
Total	3,360,405	93

Banishment of cyanide fishing in Philippines and Indonesia

Under Philippine laws, the trade of cyanide caught live reef fish is not allowed. Such laws are Republic Act 8550, "Fisheries Act of 1998"; and Republic Act 6969, "An Act to Control Toxic Substances, Hazardous and Nuclear Wastes". RA 8550 - Section 88 that prohibits the use of chemical poisoning substances in coral reefs. Under this

law, the Bureau of Fisheries and Aquatic Resources (BFAR) is the government department responsible to develop, improve, manage and conserve the country's fisheries and aquatic resources by regulating the importation and exportation of fish and aquatic products [18]. Cyanide fishing has not ceased in the Philippines, but it has certainly been reduced as a result of the governmental efforts [7, 16, 18, 19].

In the past 15-20 years the progressive deterioration of Indonesia's coral reefs have been observed [16] and the legality applied through the Indonesia territory is the Fisheries Law Act No. 31/2004 which explicitly prohibits the use of chemical substances with high toxicity at the coral reef areas. In the last several years the country had proposed to create a governmental department named Telapak's Integrated Approach to Destructive Fishing Reform, aiming to contribute to the auto-sustainability of the marine ornamental industry through an integrated approach to finally banish cyanide fishing from their islands [18].

3.1. Cyanide fishing methodology

To capture fish that seek cover in the reefs, collectors spray a solution of sodium cyanide, which effectively stuns the fish long enough to be easily collected by hand. According to the amount of solution sprayed onto the reef, the concentrations effects vary from nonlethal anaesthesia to death [7, 10].

The cyanide fishing process can be summarized by the followed steps:

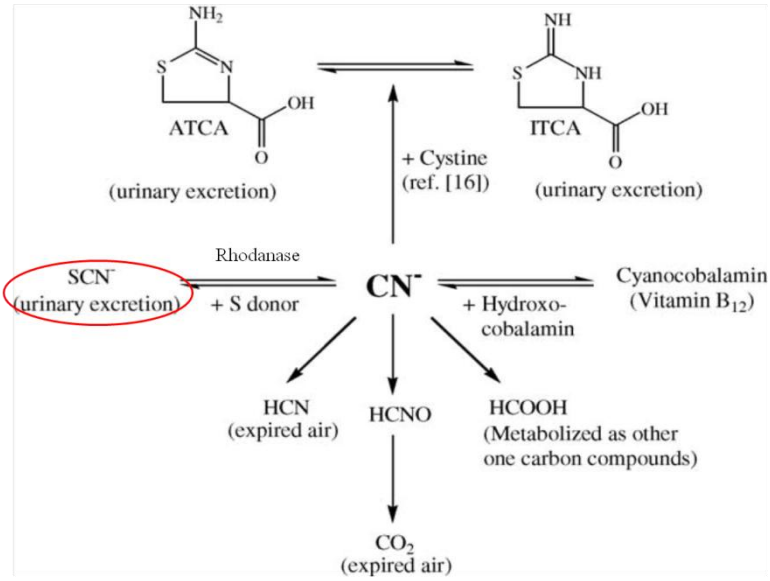
- ✓ fishermen insert (NaCN) or (KCN) tablets into squirt bottles containing seawater forming (HCN);
- ✓ they squirt hydrogen cyanide (HCN) solution into coral crevices sheltering fish;
- ✓ the cyanide temporarily stuns fish;
- ✓ stunned fish are brought to fishing boats and put into fresh seawater to recover.

Unable to control the quantity of cyanide solution that is squirted into coral reefs, as well as its concentration along the fishing period, collectors are frequently exposing themselves and the entire ecosystem to different concentrations of cyanide solution that may trigger distinct poisoning reactions. To understand those consequences and minimize these impacts, as well as to understand contamination forms and the toxicity of the chemical, it is urgent to understand the dynamics of cyanide poisoning and decontamination.

3.2. Forms of contamination and cyanide excretion process

For all animals, cyanides are well absorbed via the gastrointestinal tract or skin, and rapidly absorbed via the respiratory tract. Once absorbed, cyanide is rapidly distributed throughout the body, although the highest levels are typically found in the liver, blood, and brain. There is no accumulation of cyanide in the blood or tissues following chronic or repeated exposure, since fish exposed to cyanide naturally excrete thiocyanate as a way of self-depuration [20]. That can be explained by the transformation of cyanide into thiocyanate (SCN^-), a product metabolized in the liver by the mitochondrial sulfurtransferase enzyme rhodanese and other sulfurtransferases. Thiocyanate is excreted in the urine and the small portion of cyanide that combines with the vitamin B-12, forming the cyanocobalamin, is excreted in the urine as well [2,3],[15, 16].

Figure 1 - Conversion of cyanide into thiocyanate - Fish excretion process. Adapted from Logue et al. (2005)



This mitochondrial enzyme has already been isolated from fish gills and intestine, although it is most active in the liver and kidneys [27,28], with about 80% of all cyanide entering the organism converted to SCN- and later excreted in the urine [23]. Although this detoxification process has already been documented in freshwater fish, available information is mostly based on chronic cyanide exposure trials, rather than acute pulse dosage using high cyanide concentrations over a short period of time (similar to those employed to illegally collect reef fish with cyanide) (e.g. 30-90 s) [25,29-32]. Additionally, freshwater fish display a dramatically different strategy from marine fish on the maintenance of their osmotic balance, as they present a higher rate of urinary excretion (they have a higher blood ion concentration than surrounding water) [33, 34]. In this way, it is reasonable to assume that marine fish may retain SCN^- for longer periods than that recorded for freshwater fish [20, 35].

3.3. Toxicity effects in humans and fish

The high acute toxicity by all routes of administration present in cyanide has a highly dependent step- and rate- dose-effect curve, attributed to a chronic toxicity. These characteristics are, in general, mediated through the main metabolite and detoxification product: thiocyanate. According to IPCS (2004) the toxicity effects of cyanide in animals and humans are very similar and are believed to originate from the cytochrome oxidase inactivation, which provokes an inhibition of cellular respiration followed by histotoxic anoxia. In this process, the primary targets are the cardiovascular, respiratory and nervous systems. The endocrine system is also a potential target for a long-term toxicity, as a function of continued exposure to thiocyanate, which prevents the uptake of iodine in the thyroid and acts as a goitrogenic agent [15].

A major toxic action of cyanide is the inhibition of electron transport in the cytochrome transport chain. This occurs due to the affinity of the cyanide ion (CN^-) towards metal-centered biomolecules, and its tendency to bind

to other enzymes and molecules (e.g., methemoglobin) in order to create relatively stable, yet reversible, new complexes.

In humans, according the UPCS (2004), the main path of cyanide contamination is through the ingestion of contaminated water, or from vegetables containing cyanogenic glycosides, such as manioc and other roots, which can release cyanide during the hydrolysis process. Furthermore, the contamination via skin is also mentioned in the UPCS report. However, the cyanide absorption by the fisherman during the cyanide fishing, and the subsequent consume of poisoned cyanide fish (e.g. consummation of groupers), weren't mentioned in any of the analysed documents used as reference in this state of the art.

Rubec (1986) reported that more than 80% of the fish that survive the initial exposure to cyanide die before reaching the retailer, usually within 6 weeks of capture, suggesting possible latent cyanide toxicity [21]. However, Hanawa (1998) showed that cyanide induced anesthesia under laboratorial conditions can have minimal effects, as measured by 100% post-exposure survival rates and normal gut tissue histology [7, 10]. During pulse-exposure to cyanide the fish may not die outright, but instead may be subjected to higher risks of predation and disease. Another concern is the possible role of cyanide in contributing to the high rates of post-capture mortality in fish previously exposed to the poison [7, 10].

4. Detection methods: cyanide and thiocyanate

Cyanides in environmental media are usually collected in sodium or potassium hydroxide and measured by spectrophotometry, colorimetry, ion-specific electrode or by headspace gas chromatography with a nitrogen-specific detector or electron capture detector [16]. In aqueous material, cyanides are usually measured by colorimetric, titrimetric or electrochemical methods after pre-treatment, to produce hydrogen cyanide and absorption in sodium hydroxide solution [15]. The three commonly used techniques may suffer from interference problems, unless proper precautions are taken [15]. Most of these methods either require sophisticated operation or take long operation times. Additionally, all the methods referred, with exception of LC-UV, cannot be directly applied to the determination of thiocyanate in seawater. Therefore, there is still a need for the development of a simple, fast and reliable method for determination of SCN^- in seawater.

Cyanide-ion selective electrodes (ISE) have been successfully used to identify live reef fish collected with cyanide up to 14 days post-exposure [7] and so far have been the most successful technique in this field. In our study, optical fiber (OF) sensors, based in OF methodologies, were selected to detect trace levels of excreted thiocyanate, as they have proven to be highly sensitive, fast, less expensive and of easy operation for several analyses. Described as a non-invasive and non-destructive technique of detection for coral reef fish collected using cyanide fishing, the OF sensor was developed for detection of thiocyanate in seawater samples and validated by comparison with a high performance liquid chromatography using UV detector (HPLC-UV) methodology. Because

the OF methodology was recently developed, to thoroughly explain this technique, the work describing all the steps will be present in an attachment as the **ANNEX 1** [20].

Table 3 presents a summary of the main techniques involved at the cyanide/thiocyanate detection process.

Table 3- Detection methodologies and range limits (modified from Mak et al.,2005).

Detection Methodology	Chemical Detected	Range of detection	Authors
Optical Fiber	thiocyanate	> 3.16 µg L-1	Silva et al. (2011)
Colorimetric	cyanide	$3.8 \times 10^{-}$ to 3.8×10^{-t} M	APHA (1985), Burtis and Ashwood (1994)
ISE	cyanide	$1 \times 10^{-}$ to 1×10^{-t} M	Pohlandt et al. (1983)
Titrimetric	cyanide	$>3.8 \times 10^{-3}$ M	Bark and Higson (1963)
Gas chromatography	cyanide	$>9.5 \times 10^{-9}$ M	Way (1984)
Spectrophotofluorometry	cyanide	$>1 \times 10^{-1}$ M	Morgan and Way (1980), Guilbault (1990)
Indirect atomic absorption spectrometry	cyanide	$1.14 \times 10^{-1.}$ to 1.14×10^{-to} M	Danchik and Boltz (1970), Gomez and Calatayud (1998)
Oxygen uptake by bacteria	cyanide	$3.8 \times 10^{-}$ to 3.8×10^{-t} M 1.9×10^{-1} to 3.8×10^{-t} M	Lee and Karube (1995, 1996a) Lee and Karube (1996b)
Inhibition on yeast respiration	cyanide	LOD = 1.5×10^{-O} M; 0–1.5×10^{-M} M LOD = 3.04×10^{-LO} M	Ikebukuro et al. (1996a, 1996b) Filipovic-Kovacevic (2002)
Cytochrome oxidase inhibition	cyanide	LOD = 3.8×10^{-O} M LOD = 5×10^{-L} M	Albery et al. (1990a, 1990b) Amine et al. (1995)
Peroxidase inhibition	cyanide	LOD = 1.9×10^{-O} M	Smit and Cass (1990)
Tyrosinase inhibition	cyanide	$1 \times 10-5$ to 2.5×10^{-x} M, LOD = 2×10^{-LO} M, $2 \times 10^{-M,}$ to 4×10^{-to} M	Smit and Rechnitz (1993), Besombes et al. (1995) Hu and Leng (1995)
Heat evaluation by rhodanese	cyanide	2×10^{-x} to 1×10^{-to} M	Mattiasson and Mosbach (1977)
Rhodanese and sulfite oxidase reactions	cyanide	5×10^{-5} to 1×10^{-to} M	Groom and Luong (1991)

5. The Clownfish *Amphiprion clarkii* – Selection of the biological model

Nearly half of all live reef fish traded for the marine aquarium industry belong to the family Pomacentridae (damselfish) [22] and the use of cyanide to collect species from this family has already been recorded [23]. *Amphiprion clarkii*, a species member of genus *Amphiprion* (one of the two genus of clown fish) was selected as a model for our work since it is one of the most popular fish among marine aquarium hobbyists [22] and widely spread in Indo-Pacific reefs [24, 25]. Aiming to understand how long thiocyanate depuration can last without any other interference, the specimens used in our experiment were purchased from a local marine ornamental fish producer in Portugal in order to assure that tested specimens had never been previously exposed to cyanide.

A brief resume of *A. clarkii* biology and specifics ecology aspects, as the general captive maintained conditions, will be presented in order to better understand the biology model adopted for our work.

Figure 2 - Taxonomic classification and Natural distribution of *A. clarkii* – Major occupation area

Taxonomy classification

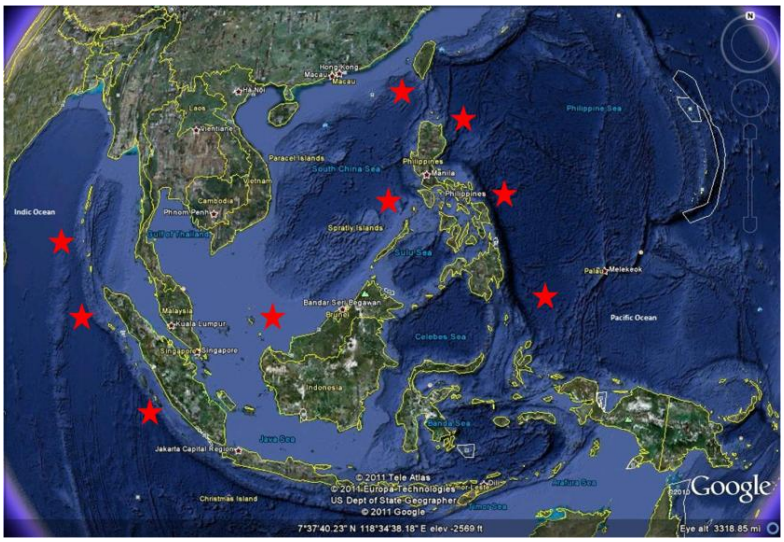
Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Pomacentridae



Natural geographic location: the Banded Clown fish or Clark's Clown fish (Clark's Anemonefish) *Amphiprion clarkii* was first described by Bennett in 1830. This is the most widely distributed anemonefish ranging from the islands of Micronesia and Melanesia in the western Pacific to the Persian Gulf and from Australia to Japan. Throughout the Indo-Pacific, along the islands of Leyte and Cebu, in the central Philippines, *A. clarkii* populations are continuously spared, except where coral reefs are disrupted by sandy sediment near major river outflows [11, 24, 26, 27].

Conservation status: the species is not listed on the IUCN Red List [28].

Feeding habits: Omnivores.

Morphology description: the boldly patterned Clark's Clownfish can be quite diverse in colour as an adult, presenting yellow to brown colour variations with either two or three white to grey bands. All juveniles develop a yellow tail, which changes to white in females as they mature [22, 29]. In this specie, large fish are aggressively dominant over smaller members in a group, where the largest two fish usually control the growth and maturation of subordinates. Adult males are smaller than females and they are described as a group with monogamous mating system, maintained by a protandrous sex reversal system, in which only the two largest of each group can spawn in a certain area [30]. The inhibitory behaviour is also observed in the inhibition of juvenile growth by adults, since a non-breeding juvenile becomes a breeding adult only if either one or both of the mated adults disappear from their territory [30].

Social Behaviours: Clark's Clownfish are small and territorial, mainly inhabiting coral reef regions, and always live on or around host anemones that are an essential resource for their shelter and spawning sites [3]. In these symbiotic relationships, clown fish and sea anemones live together, each benefiting from the other's company, forming a mutualistic association. As they get older, the specimens can become more territorial and aggressive. Hattori (2005) confirmed that when the *A.clarkii*, as the Alfa-specie in the cohabiting group, become larger in size and aggressively dominant over *A.perideraion*, they suppress the growth and reproduction of this specie. Probably, the characteristic of A. *clarkii* as pioneer is related closely to its wide range of host anemone species; consequently, the distribution of the pioneer species is widespread from tropical coral reefs to temperate rocky reefs [3].

Captive maintenance: Clark's Clownfish can be kept captive in saltwater aquariums and still associate with anemones in these artificial habitats. *A. clarkii* is not only a good disperser, considering its capacity to find a vacated host, but also a pioneer species that is able to use newly settled small hosts [3]. Larval *A. clarkii* settle on such a small host because they able to move to larger hosts for future reproduction. In contrast, the higher colonization success of *A. clarkii* after habitat destruction seems to be caused by their small host utilization ability. As mentioned earlier, small host utilization ability is related closely to body size and fish mobility. The body size difference between competitors seems to play an important role in their coexistence in a patchy environment. They are very active and need an open space for free swimming [3] but in our experimental model we used: water temperature between 21 - 26° C, salinity between 30 and 35, pH 8.1 ± 0.1, ammonium and nitrite not detectable, and nitrate below 5 mg L^{-1}.

Research Goal

As was mentioned above, marine ornamental fish are valuable organisms and any method requiring the sacrifice of the fish to detected cyanide are not well received by the traders market. Based on the OF methodology, the main goal of our M.S.Project was to prove that OF sensor is able to detect in real time trace levels of excreted SCN^- in a non invasive way. By analysing the water of the container holding the fish and not the fish tissues, as the other technologies does. Following this trend, we validated this inexpensive methodology in laboratory and the methodologies adopted as the experimental details and obtained results, as well, are described at the Chapter 2.

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Chapter 2

Excreted SNC- Detects Cyanide Caught Fish

Chapter 2

Excreted SNC^- Detects Cyanide Caught Fish

EXCRETED THIOCYANATE DETECTS LIVE REEF FISH ILLEGALLY COLLECTED USING CYANIDE – A NON-INVASIVE AND NON-DESTRUCTIVE TESTING APPROACH

SHORT TITLE

Excreted Thiocyanate Detects Cyanide Caught Fish

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TAPR-S IL RP PJR RC. Performed the experiments: MCMV RJMR IL RP RC. Analyzed the data: MCMV TAPR-S AD RC. Contributed reagents/materials/analysis tools: TAPR-S IL RP AD RC. Wrote the paper: MCMV PJR RC.

COMPETING INTEREST

The authors have no financial, personal, or professional interests that could be construed to have influenced this paper

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ABSTRACT

Cyanide fishing is a method employed to collect live marine fish on coral reefs. They are either shipped to markets for human consumption in Southeast Asia or to supply the marine aquarium trade worldwide. Although several techniques can be used to detect cyanide in reef fish, there is still no testing method which can be used to survey the whole supply chain. Most methods for cyanide detection are time consuming and require the sacrifice of surveyed fish. Thiocyanate anion (SCN^-) is a metabolite produced by the main metabolic pathway for cyanide anion (CN^-) detoxification. Our study employed an optical fiber (OF) methodology (analytical time < 6 min) to detect SCN^- in a non-invasive and non-destructive manner. Our OF methodology is able to detect trace levels (> 3.16 µg L^{-1}) of this compound in seawater. Given that marine fish exposed to cyanide excrete SCN^- in the urine, abnormal levels of SCN^- present in the seawater holding live reef fish can indicate that the surveyed specimens were most likely exposed to cyanide. In our study, captive-bred clownfish (*Amphiprion clarkii*) pulse exposed during 60 s to either 12.5 or 25 mg L^{-1} of CN^- excreted up to 6.96 ± 0.03 and 9.84 ± 0.03 µg L^{-1} of SCN^-(respectively) during 28 days following exposure. No detectable levels of SCN^- were recorded in the water holding control organisms (not exposed to CN) or in batches of prepared synthetic seawater. While further research is necessary, our methodology can allow the rapid detection of the presence of SCN, which indicates that live reef fish were collected with cyanide.

INTRODUCTION

Coral reefs are some of the most biologically rich and productive regions on our planet, providing valuable benefits to millions of people worldwide. However, these ecosystems currently face an increasing number of threats (e.g. coastal development, agricultural runoff, overfishing…) [31-34]. These negative impacts are being magnified by the effects of global change and climate change (e.g. coral bleaching induced by increasing water temperatures and ocean's acidification due to increasing carbon dioxide emissions) [35, 36].

Coral reefs in Indonesia and the Philippines are currently the ones most at risk (about 95% of existing reefs) due to the use of destructive fishing techniques [37, 38]. In ranking destructive fishing techniques, cyanide fishing is probably only second to blast fishing. Millions of fish are collected illegally every year using

cyanide from coral reefs in Indonesia and Philippines [39-41]. Highly priced groupers are either shipped live for human consumption to Hong Kong and other Asian countries [40, 42], while other marine fish are transported mainly to the USA, EU, and Japan to supply the marine aquarium trade [22].

Cyanide fishing is an inexpensive and highly effective method used to stun fish and can be described as follows: 1) fishermen insert sodium cyanide (NaCN) or potassium cyanide (KCN) tablets into squirt bottles containing seawater; 2) they squirt hydrogen cyanide (HCN) solution into coral crevices sheltering fish; 3) the cyanide temporarily stuns fish (many die from acute doses); and 4) stunned fish are brought to fishing boats and put into fresh seawater to recover [43]. It is estimated that over 500 metric tons of cyanide may be used annually to collect live fish on Philippine reefs alone [39].

Cyanide is known to be extremely destructive to marine organisms, killing both targeted and non-targeted specimens [44]. It is commonly claimed that cyanide caught fish die in transit due to their weakened condition, thus requiring fishermen to capture more organisms than would otherwise be required to supply the trade because they need to account for a fatality margin [22, 45]. Marine invertebrates are also vulnerable to cyanide poisoning, namely reef building corals. Despite leaving the reef physically intact (although corals can be broken to extract stunned fish), cyanide kills coral polyps by disrupting their symbiotic association with the zooxanthellae [46, 47]. This disruption promotes bleaching and causes irreparable losses [44]. Despite being technically illegal in most exporting countries, cyanide fishing is still a common practice, and it is often encouraged by corrupt authorities taking advantage of poor rural communities [48].

Cyanide-ion selective electrodes (ISE) have been successfully used to identify live reef fish collected with cyanide up to 14 days post-exposure [23], although several other methods are currently available to detect cyanide or cyanide metabolites (e.g. titrimetric, colorimetric and chromatographic methods, spectrophotofluorometry, enzyme-based biosensors or biomarker approaches) [49-52]. The concentrations of cyanide (or its metabolites) present in living fish tissues, blood, or urine may vary among different sized specimens and between species, as well as with the following conditions: 1) concentration of the cyanide solution used during their capture; 2) duration of the exposure period; 3) post-collection handling; 4) stocking time on exporting or importing facilities; and 5) shipping duration [53]. However, the current gaps of knowledge on the cytokinetics of cyanide and its major metabolites in marine fish impairs researchers' ability to identify the most appropriate cyanide testing methods that can be applied along the whole supply chain (e.g. collection sites, exporting and importing facilities) [53]. Live reef fish are highly priced goods, with some species reaching market values of several hundred Euros, either on the live food fish market or the marine aquarium trade. Therefore, any method requiring the sacrifice of these organisms to detect cyanide would not be welcome by most traders. Consequently, it is preferable to find a reliable method using a non-invasive and non-destructive approach to determine whether live reef fish were collected with cyanide.

It is known that the major pathway for cyanide metabolism is the conversion of cyanide (CN^-) to thiocyanate (SCN^-), in the presence of a sulphur donor, by the enzyme rhodanese (thiosulphate: cyanide

sulfurtransferase; EC 2.8.1.1) [54]. This mitochondrial enzyme has already been isolated from fish gills and intestine, although it is most active in the liver and kidneys [55, 56], with about 80% of all cyanide entering the organism being converted to SCN^- and later excreted in the urine [51]. Although this detoxification process has already been documented in freshwater fish, available information is mostly based on chronic cyanide exposure trials, rather than acute pulse dosage using high cyanide concentrations over a short period of time (similar to those employed to illegally collect reef fish with cyanide) (e.g. 30-90 s) [53, 57-60]. Additionally, freshwater fish display a dramatically different strategy from marine fish on the maintenance of their osmotic balance, as they present a higher rate of urinary excretion (they have a higher blood ion concentration than surrounding water) [61, 62]. In this way, it is reasonable to hypothesize that marine fish may retain SCN^- for longer periods than that recorded for freshwater fish [23, 63].

If the above hypothesis on the extended retention of SCN^- in marine fish after their exposure to cyanide is valid, it should be possible to detect live reef fish collected illegally by employing a sensor capable of detecting aqueous concentrations of SCN^- in containers used to hold/ship these organisms. The rationale for this approach is that fish specimens will excrete urine contaminated with SCN^- into the water of holding tanks or plastic shipping bags, leading to increasing concentrations of this compound in seawater. This approach presents the following advantages: 1) it tests for a longer lasting compound (SCN^-, rather than CN); 2) it delivers results much faster than current techniques employed for testing total CN^- (which are time consuming and labour intensive); 3) by being non-invasive and non-destructive (no need for taking fish muscle or blood samples), it does not require either the sacrifice or manipulation of the fish; and 4) it is easier, safer and cheaper to use than current techniques available to detect cyanide in fish.

The objective of the present study was to determine: 1) if abnormal levels of SCN^- can be determined non-invasively and non-destructively, by testing the seawater used during the shipping/holding of live reef fish, using a custom made optical fiber methodology (indicating the excretion of this compound and, thus, the exposure of fish to CN^-); 2) how long after pulse exposure to cyanide do reef fish start to excrete SCN^- in detectable levels; and 3) if fish continue to excrete SCN^- in detectable levels at least 4 weeks after their pulse exposure to CN^-.

MATERIALS AND METHODS

Selection of the fish model species and husbandry

Nearly half of all live reef fish traded for the marine aquarium industry belong to the family Pomacentridae (damselfish) [22]. The use of cyanide to collect Pomacentridae has already been recorded [23], thus it was reasonable to select a species of this diverse family as a model for the present work. Nonetheless, in order to assure that selected specimens had never been previously exposed to cyanide, two possible options were available: 1) select a model species whose specimens could be collected from the wild, namely from regions with no previous records of cyanide fishing; or 2) select a model species whose specimens could be

cultured in captivity and thus assure that they never had been exposed to cyanide. As traceability in the trade of live reef fish is far from being reliable [64], the first option was discarded and the use of cultured specimens in captivity was considered as the most suitable approach for the present work. Members of the genus *Amphiprion* (one of the two genus of clownfish) are some of the most popular species among marine aquarium hobbyists [22] and, comparatively to other damselfish, can be easily cultured in captivity [64, 65]. Recently, local marine aquarium retailers have reported the occurrence of high mortalities on imports of wild clownfish, namely Clark's clownfish *Amphiprion clarkii* (Bennett, 1830), and have attributed these deaths to cyanide poisoning. Although these reports are mostly anecdotal, it was decided to select *A. clarkii* as the model fish species for the present study.

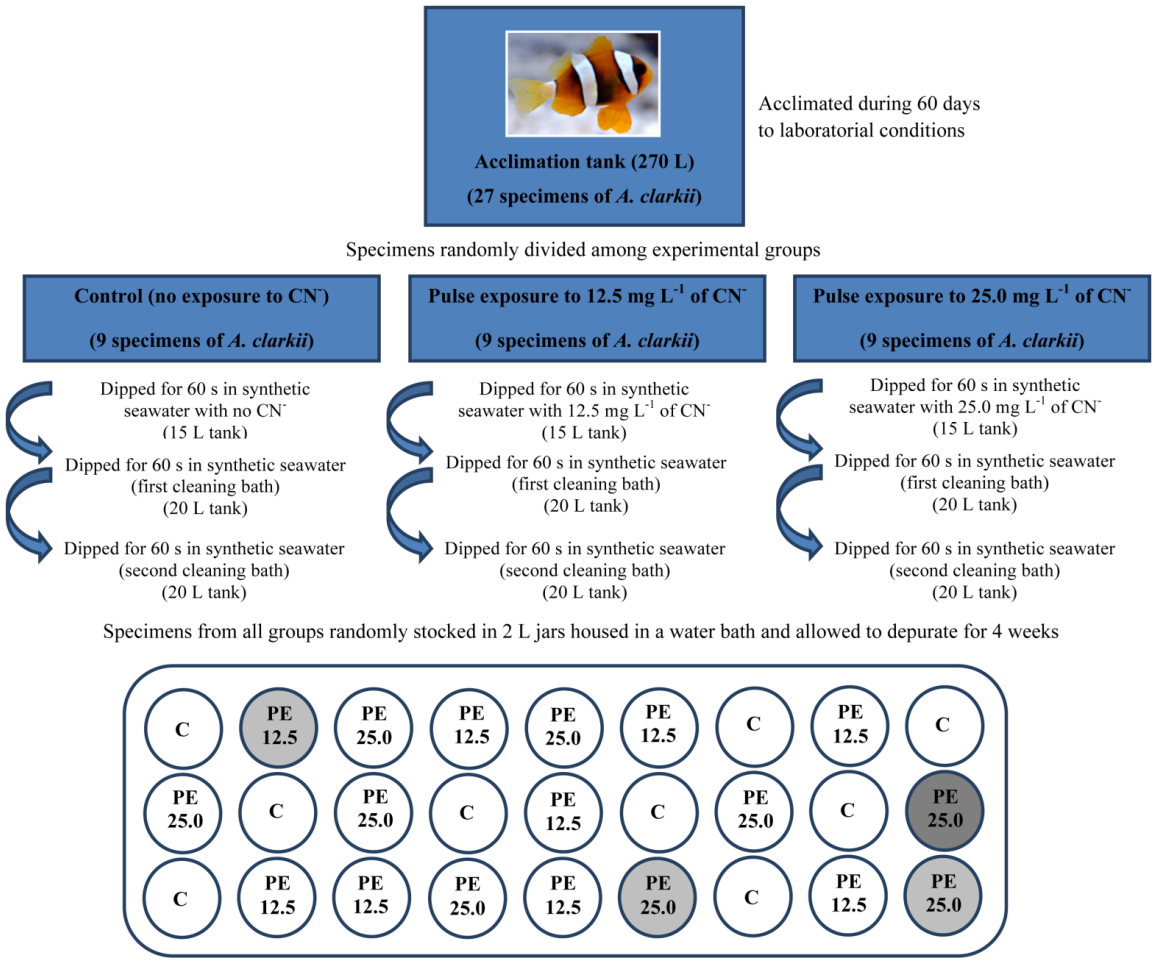
Twenty seven cultured specimens of *A. clarkii* (average total length ± standard error, SE, measured from the tip of the snout to the tip of the longer lobe of the caudal fin) of 40.3 ± 3.1 mm and average wet weight (± SE) of 1.8 ± 0.2 g were purchased from a local producer (Opérculo Lda., Portugal), thus assuring that all specimens to be used in this work had never been previously exposed to cyanide. All specimens were kept during 60 days for acclimation to laboratorial conditions in a 270 L glass tank (1.20 m long, 0.45 m wide and 0.50 m high) equipped with an internal circulation pump (Turbelle® nanostream - 6025, Tunze®, 2500 L h^{-1}) and a HQI lamp (150 W, 10.000 K, Sylvania®) providing a 12 h L:12 h D photoperiod. The glass tank was connected to a 125 L sump (1.20 m long X 0.35 m wide X 0.40 m tall) equipped with a recirculating water pump (Eheim® 1262, 3400 L h^{-1}), a 50µm mesh bag for mechanical filtration, a Deltec® APF600 protein skimmer, a biological filter (composed by submerged bio-balls) and a 300 W submersible heater (Eheim® Jäger) keeping temperature stable at 26 ± 0.5 ºC. This system was also equipped with an osmoregulator (Reef Set®), which was responsible to regulate the water level by compensating evaporated water with freshwater purified by a reverse osmosis and maintaining salinity at 35. The fish stocking system was run using synthetic seawater prepared by mixing freshwater purified by a reverse osmosis unit with Tropic Marin Pro Reef salt (Tropic Marine®, Germany). Water quality parameters were monitored every other day using colorimetric tests (Salifert®) and remained within optimal ranges for *A. clarkii* (pH 8.1 ± 0.1 ; ammonium and nitrite were not detectable, while nitrate was recorded below 5 mg L^{-1} expressed as N). All fish were fed daily to satiation with the commercial pelleted feed Hikari Marine S (Hikari®, Japan).

Cyanide pulse exposure and depuration

After the acclimation period (60 days), the 27 *A. clarkii* were randomly divided into 3 groups of 9 fish each for cyanide pulse exposure. The first group was used as a control (not being exposed to cyanide), with the second group being exposed to a concentration of 12.5 mg L^{-1} of CN^- and the third group being exposed to a concentration of 25.0 mg L^{-1} of CN^-. The pulse exposure to CN^- was performed according to the three following steps: 1) exposure bath; 2) first cleaning bath; and 3) second cleaning bath. The exposure bath was performed as follows: all fish from the same group were collected with a hand net and dipped during 60 s in a 15 L tank filled with: synthetic seawater with no cyanide (group 1); synthetic seawater contaminated with CN^- at a concentration of 12.5 mg L^{-1} (group 2); and synthetic seawater contaminated

with CN^- at a concentration of 25.0 mg L^{-1} (group 3). After the pulse exposure to cyanide all fish from the same group were dipped during 60 s into a 20 L tank filled with synthetic seawater (no cyanide) (first cleaning bath). This procedure was repeated one more time for all fish from all groups (second cleaning bath) (see Figure 3 for a schematic representation). The duration of the pulse exposure to CN^- (60 s) was selected according to the work by Hanawa et al. [66]. The concentrations of CN^- initially selected for the pulse exposure to this poison in the present study were also those described by Hanawa et al. [66] (25.0 and 50.0 mg L^{-1}), as these concentrations were considered to simulate well in field conditions. However, since a preliminary experiment revealed that at 25.0 mg L^{-1} of CN^- some fish mortality already occurred after an exposure of 60 s, it was decided to select 12.5 and 25.0 mg L^{-1} (rather than 25.0 and 50.0 mg L^{-1} of CN^-) as the experimental concentrations to be tested during pulse exposure. Two different chemicals are commonly employed in cyanide fishing: sodium cyanide (NaCN) and potassium cyanide (KCN). As both chemicals are potent poisons, it has been assumed that their fish stunning capacity may not differ significantly [67]. In order to allow a better comparison of experimental results, the present work selected the same chemical as Hanawa et al. [66], that is NaCN. A stock solution of 18.84 g L^{-1} of NaCN (97% purity; Sigma-Aldrich, St. Louis, MO, USA), with a CN^- concentration of 10.00 g L^{-1}, was prepared by dissolving 5.65 g of NaCN in 300 mL of Milli Q water. The experimental concentrations of CN^- employed during the pulse exposure (12.5 and 25.0 mg L^{-1}) were prepared by adding 18.7 mL and 37.5 mL of the stock solution to 15 L of synthetic seawater, respectively.

Figure 1 - Schematic representation of experimental procedures for cyanide (CN^-) pulse exposure and depuration of *Amphiprion clarkii*.



Following the pulse exposure to CN^-, all fish were individually randomly distributed into 2 L glass jars (0.12 m in diameter and 0.25 m tall) filled with 1.5 L of synthetic seawater for post-exposure depuration (see Figure 3 for a schematic representation). All glass jars were equipped with an air stone to provide gentle water aeration, placed inside a water bath keeping water temperature at 26 ± 0.5 ºC and exposed to a photoperiod of 12 h L:12 h D provided by white fluorescent lamps. The behavior of all fish was monitored during the first 30 minutes after being stocked inside the glass jars, in order to record recovery and mortality promoted by cyanide pulse exposure. Clown fish were allowed to depurate for a 4 week period, as this time window overlaps with the period commonly elapsing between the collection of a marine ornamental fish and its purchase in retail stores by hobbyist [66]. It must also be highlighted that traders selling cyanide caught fish will try to minimize the holding period of these specimens in order to reduce the risk of poisoned fish to die in their facilities. Therefore, it is likely that cyanide caught fish will be shipped sooner to importing wholesalers and retailers than net caught specimens. During the depuration period the water from each jar was fully replaced every day after feeding stocked fish to satiation with the commercial pelleted feed described above. A water sample of 1 mL was collected daily with a micropipette from each

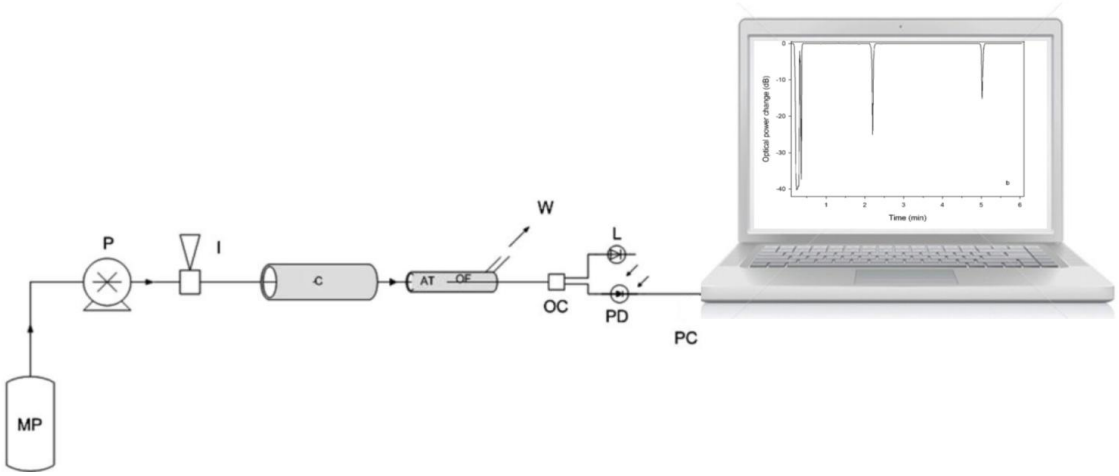
jar prior to feeding and stocked at -20 ºC for posterior determination of SCN^- levels. Each time a new batch of synthetic seawater was prepared (including the seawater used to prepare the solution for pulse exposure to CN^-, as well as cleaning baths) water samples were also collected in order to determine any background levels of SCN^-.

C – control specimens not exposed to CN-; PE 12.5 – Specimens pulse exposed during 60 s to 12.5 mg L-1 of CN-; PE 25.0 - Specimens pulse exposed during 60 s to 25.0 mg L-1 of CN-; Circles with a white background represent specimens surviving until the end of the depuration period, while light and dark grey backgrounds represent specimens dying at least 30 min after the pulse exposure and during the depuration period, respectively.

Thiocyanate analysis

The concentration of SCN^- present in the water samples collected during the experimental period were determined using a new methodology based on optical fiber (OF) detection coupled to a liquid chromatography system (see Figure 4 for a schematic representation and S1) [68]. This new technique displays a high analytical performance, both in terms of linear range (4-400 µg L^{-1}) and detection limits (3.16 µg L^{-1}), an analytical time of less than 6 min and is comparable to methodologies employing high performance liquid chromatography with UV detector (HPLC-UV) [68]. This new OF methodology is an excellent platform for fast and inexpensive analysis of SCN^- content in seawater samples, thus being ideal for detecting abnormally high levels of this compound on the water holding live coral reef fish (indicating their collection using cyanide).

Figure 2 - Schematic representation of the OF based methodology



MP - mobile phase flask; P - pump; I - injector; C - column; AT - analytical tube; OF - optical fiber; W - waste; OC - optical coupler; L - laser diode optical source; PD - photodiode detector; PC - laptop with homemade

software displaying the analytical signal obtained by OF based methodology for a sample of seawater with thiocyanate (SCN^-) standard addition.

Statistical analysis

The existence of significant differences in the levels of SCN^- in the water samples collected along the duration of the experiment (28 days) were analyzed using a repeated measurements ANOVA, with the concentration of cyanide employed during the pulse exposure used as the categorical factor. Statistical analyses were performed using the software STATISTICA version 8.0 (StatSoft Inc.), with the assumptions of normality and homogeneity of variance checked prior to analysis through the Shapiro-Wilks and Levene test, respectively. Mauchly's test of sphericity was used to determine if the variances of the differences between all combinations of related groups (levels) are equal. Whenever significance was accepted, at $p < 0.05$, the Tukey multiple comparison test was used for pairwise comparison of means [69].

Ethics Statement

This study was carried out in strict accordance with the recommendations present in the Guide for the Care and Use of Laboratory Animals of the European Union – in Portugal represented by the Decreto Lei n˚129/92 de 06 de Julho, Portaria n˚1005/92 de 23 de Outubro de 1992. Approval by a named review board institution or ethics committee was not necessary as the final model for ethical experimentation using fish as biological models is yet to be implemented in Portuguese research units.

RESULTS

Fish anesthesia and mortality following pulse exposure to cyanide

During the pulse exposure to CN^- at both tested concentrations all fish displayed frantic swimming and strong gasping behavior, followed by a loss of balance, cessation of swimming (specimens rested motionless at the bottom of the container) and finally a complete loss of respiratory activity (after about 30 and 50 s of the beginning of the pulse exposure with 12.5 and 25.0 mg L^{-1} of CN^-, respectively). One and two specimens of *A. clarkii* exposed to 12.5 and 25.0 mg L^{-1} of CN^-, respectively, did not recover from anesthesia and died within the next 30 minutes following pulse exposure. Apart from a more agitated swimming behavior due to the netting during the 3 steps of the pulse exposure, fish in the control group did not display any of these responses and no mortality was recorded.

Fish mortality and thiocyanate excretion during depuration

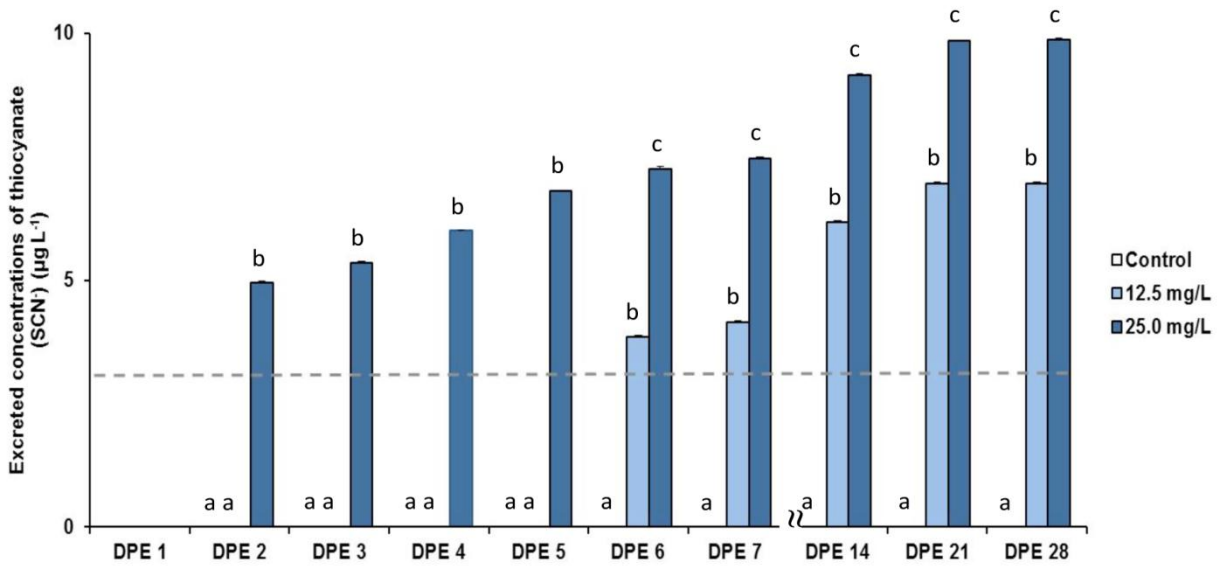
A single specimen of *A. clarkii* died during the depuration period at day 18 post-exposure (a fish previously exposed to 25.0 mg L^{-1} of CN^-). The total number of *A. clarkii* reaching the end of the depuration process (28

days) were 9, 8 and 6 specimens respectively for the control group, and the groups pulse-exposed to 12.5 and 25.0 mg L^{-1} of CN^-.

During the whole depuration process no detectable levels (> 3.16 µg L^{-1}) of SCN^- were ever recorded in the water samples collected from the jars holding fish from the control group (no exposure to CN^-) (Figure 5). On the first day post-exposure (DPE), no detectable levels of SCN^- were recorded in the water samples collected from jars stocking fish pulse exposed to CN^-. However, on the second DPE an average concentration (± standard error) of 4.95 ± 0.04 µg L^{-1} of SCN^- was already detected in the water holding *A. clarkii* pulse exposed to 25.0 mg L^{-1} of CN^-. Concerning *A. clarkii* pulse exposed to 12.5 mg L^{-1} of CN^-, only 6 days after exposure were SCN^- levels recorded above the detection limit of our OF methodology (average content of 3.84 ± 0.04 µg L^{-1}). In that same period, water samples collected from specimens pulse exposed to 25.0 mg L^{-1} of CN^- displayed a significantly higher content of SCN^- (7.25 ± 0.05 µg L^{-1}) ($p < 0.0001$) (Figure 5). By the end of the third depuration week (21 DPE) SCN^- levels in the water had increased to 6.96 ± 0.03 and 9.84 ± 0.03 µg L^{-1} for fish pulse exposed to 12.5 and 25.0 mg L^{-1} of CN^-, respectively, and remained significantly higher in those specimens being pulse exposed to a higher concentration of CN^- ($p < 0.0001$). When the experiment was ended (28 DPE) SCN^- levels in the water stocking fish pulse exposed to 12.5 and 25.0 mg L^{-1} of CN^- had remained nearly identical to those recorded at 21 DPE (Figure 5).

No detectable levels of SCN^- (> 3.16 µg L^{-1}) were ever recorded in the samples collected from the batches of newly prepared synthetic seawater.

Figure 3 - Concentrations (µg L^{-1}) of thiocyanate (SCN^-) excreted during the depuration period of *Amphiprion clarkii*.



Values are averages ± standard error of SCN^- excreted by fish not exposed to cyanide (control group) or pulse exposed to 12.5 or 25.0 mg L^{-1} of cyanide. The grey dashed line represents the detection limit (3.16 µg

L^{-1}) of the optical fiber methodology employed to determine SCN^- concentrations in the water samples collected from the jars holding *A. clarkii* during the depuration period. Different superscript letters in columns on the same day post-exposure (DPE) represent significant differences at $p < 0.05$.

DISCUSSION

The present work showed that our OF methodology could easily be used to determine abnormal concentrations of SCN^- in seawater samples collected from containers holding live fish previously pulse exposed to CN^-. This non-invasive and non-destructive approach, with an analytical time of less than 6 min [68], allowed us to monitor accurately, during a 4 week period, the daily excretion of SCN^- by poisoned fish. However, our results also showed that SCN^- excretion varies according to the initial concentration of CN^- during pulse exposure, as well as with the number of DPE after which the water samples are collected. While detectable levels of SCN^- excreted by poisoned fish were recorded in the next day following a pulse exposure to a given concentration of CN^- (e.g. 25.0 mg L^{-1} of CN^-), it took nearly one week to detect cyanide caught fish through the analysis of excreted SCN^- in water samples if those specimens had been collected using a lower concentration of CN^- (e.g. 12.5 mg L^{-1} of CN^-). Apart from the initial concentration of CN^- during pulse exposure, there are several other aspects that may influence the excretion of SCN^- by poisoned fish, namely: 1) duration of the exposure period, 2) fish species and size, 3) collection and post-collection handling stress, 4) stocking time and husbandry on exporting or importing facilities and 5) shipping duration [23, 53, 66]. There is some available information on the initial concentrations of CN^- on apprehended squirt bottles employed for cyanide fishing (ranging from 760 to over 2000 mg L^{-1}) [70]. However, the absolute concentrations to which fish are exposed in the field can only be estimated. In this way, it is nearly impossible to predict how soon marine fish collected with cyanide will start to excrete SCN^- in detectable levels by our FO methodology. Our work in comparison to that of Hanawa et al. [66] may indicate that fish species and size can influence the effects of CN^- pulse exposure, as a concentration and exposure time that promoted no mortality in *Dascyllus aruanus* (25.0 mg L^{-1} of CN^- and 60 s, respectively) was lethal for some of our specimens of *A. clarkii* with slightly smaller sizes. If such physiological variability can occur among members of the same family (both *D. aruanus* and *A. clarkii* belong to the Pomacentridae [71]) and cyanide poisoning has already been recorded in at least 36 different reef fish families [23], a significant interspecific variation might also be expected in the post-exposure metabolization of CN^- and excretion dynamics of SCN. An aspect that has already been shown to promote mortality in marine fish after their pulse exposure to CN^- is handling stress [66, 72]. Although our study has not considered this variable, it is possible that handling stress may also interfere with the excretion of SCN^-, as it is suspected that it may promote osmoregulatory dysfunction [72]. However, given our current lack of knowledge on CN^- metabolism in fish [51] it is impossible to determine whether the excretion of SCN^- will increase or decrease with handling stress.

The stocking systems on which collected specimens are held before shipping to importing countries, as well as those employed in importing countries to stock live reef fish, may also interfere on the excretion of SCN^- of fish collected with CN^-. Most traders employ recirculated holding systems (the water is filtered by life support systems employing, among other components, biological filtration and protein skimming) [73], while the present study employed glass jars with a relatively small volume to stock cyanide poisoned fish, with their water volume being fully replaced every day by clean seawater.

Our study has not allowed us to determine how long poisoned fish will continue to excrete SCN^- in detectable levels, as depuration was interrupted 28 DPE. Nonetheless, our results clearly showed that SCN^- excretion in a marine fish does not occur as fast as in freshwater fish [57] and agree with the findings by Rubec et al. [23] on the prevalence of CN^- on marine fish tissues for up to 3 weeks after collection. Additionally, our study also supports the assertion of Rubec et al. [63] which claimed that marine fish do not quickly metabolize CN^- and excrete SCN^- in a matter of hours, as the lower rate of urinary excretion of marine fish (in comparison to freshwater fish) promotes the retention of SCN^- for longer periods.

By increasing the holding period in export facilities of fish beyond the end-point of SCN^- excretion, it might be possible to avoid detection by our OF methodology for fish imported and tested in the EU or in the USA. However, when trading cyanide-caught fish, it is a common practice along the chain-of-custody to sell them as fast as possible, in order that any losses occur in the next link of the chain (ultimately the marine aquarium hobbyist). By stocking cyanide-caught fish for longer periods wholesalers and/or retailers risk losing the fish due to their high mortality [39]. They could also be subject to prosecution, since law enforcement officials will be able to detect SCN^- in the holding tanks (as they would be holding them during the excretion period of SCN^-).

If cyanide-caught fish are held with net-caught fish in export facilities, it may be possible for the net-caught fish to take up SCN^- [68, 74]. While it is unlikely that this would contaminate fish to the extent that they would excrete detectable levels of SCN^- there is a chance that if a net-caught fish is shipped in holding water from such export facilities, it can be erroneously considered by our OF methodology has being cyanide caught. Traders acting in good faith can avoid this issue simply by shipping their specimens in synthetic seawater (such as in the present work), as it will not present any detectable levels of SCN^- (at least by our OF methodology). Enforcement personnel in importing countries can avoid this situation on import simply by isolating the target fish to be tested during 24 h in a container filled with artificial seawater (e.g. during the quarantine period) and only then employ our OF methodology to detect excreted SCN^- (water samples from the artificial seawater employed to stock the specimen could be used as a control).

In conclusion, our OF methodology has proven to allow a fast and accurate determination of trace SCN^- levels in seawater (> 3.16 µg L^{-1}) and can reveal the detection of live reef fish collected illegally using cyanide in a non-invasive and non-destructive approach. In the future it is possible that this approach can be used to screen live reef fish immediately upon arrival to importing countries, taking advantage that in most situations imported specimens have been confined in the same seawater for several hours (thus increasing the chances of detecting abnormal levels of excreted SCN^-). In importing countries, it can be

easier to act legally against traders landing cyanide caught fish and the authorities of importing countries can also provide information to exporting countries on which traders are supplying these illegally collected organisms. This strategy may discourage importing enterprises to buy from unreliable suppliers and shift the legal pressure from impoverished fishermen trying to feed their families to those truly profiting with this illegal and destructive activity.

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Chapter 3

Concluding Remarks and Future Steps

Chapter 3

Concluding remarks and Future Steps

Mainly shipped to the markets of USA, EU, and Japan, to supply the marine aquarium trade as well as to meet high human consumption demands in Hong Kong and other Asian countries, live reef fish are highly priced goods. However, despite being illegal in most exporting countries, cyanide fishing is still a common reef fishing technique around the world. Benefiting from the inexistence of a suitable technology to determine if traded fish have been poisoned with cyanide, most cyanide and thiocyanate detection techniques currently available take a long time to be processed in this field. Furthermore, any method requiring the sacrifice of these organisms in order to detect cyanide would certainly not be welcome by traders. Consequently, the use of a reliable method using a non-invasive and non-destructive approach to determine whether live reef fish were collected with cyanide is extremely necessary. To that end, the work presented in this thesis has shown that the OF methodology can detect trace levels of excreted SNC^-, proving it possible to detect live reef fish collected with cyanide following these advantages:

- ✓ it tests SCN- instead CN-;
- ✓ the results obtained are much faster than current techniques employed for testing total CN^-;
- ✓ because is a non-invasive and non-destructive technique (no need for taking fish muscle or blood samples), it does not require either the sacrifice or manipulation of the fish;
- ✓ it is easier, faster, safer and cheaper to use than current techniques available to detect cyanide in fish.

Following these trends raises the possibility of stimulating corrupt authorities to stop taking advantage of poor rural communities from Indo-Pacific Ocean areas, as they are the ones commonly supplying cyanide for fishing purposes. If well developed, this methodology can also allow fish to be analysed at the border, right before being shipped or landed abroad, taking advantage that in most situations imported specimens have been confined in the same seawater for several hours. In importing countries, it can be easier to act legally against traders landing cyanide caught fish and the authorities of importing countries can also provide information to exporting countries on which traders are supplying these illegally collected organisms. These strategies may discourage importing enterprises to buy from unreliable suppliers and that can be a good tactic to finally preserve the coral reefs at the Indic-Pacific areas.

Aiming to make this methodology faster and more efficient in the future, we also suggest that the OF sensor become smaller, portable, equipped with wireless internet and/or bluetooth to create an ideal communication with a central computer storing and interpreting all collected data from the shipped

organisms; and the investigation of other aspects that may influence the excretion levels of SCN^- from poisoned fish, as:

- ✓ the variability of size, stage of life, species and families analyzed;
- ✓ the effects of the exposure period according to the concentration of the chemical pulse-exposure;
- ✓ the effects of the collection and post-collection handling stress at the SNC- values.

We further propose exporting, importing and shipping methodologies to standardize procedures, enabling detection of the real values of SNC- during these processes, and to compare the obtained results.

Annex-1

PDF file of the manuscript describing in detail the methodology employed in this work to determine thiocyanate (SCN^-) in seawater.

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PAPER

Optical fiber based methodology for assessment of thiocyanate in seawater

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A new methodology for the assessment of thiocyanate (SCN^-) is proposed based on optical fiber (OF) detection coupled to a liquid chromatography system (LC). The developed methodology showed an adequate performance for the analysis of SCN^- comparable to a high performance liquid chromatography with UV detector (HPLC-UV) methodology: a detection limit of 3 μg L^{-1}, a linear range from 4 to 400 μg L^{-1}, and an analytical time of less than 6 min. The OF based methodology was of compact design and easy operation. This simple system has the potential to be used as a sensing approach for SCN^- in seawater.

Introduction

Thiocyanate (SCN^-) has been widely used in fabric dyeing, photography and electroplating industry,[1] and it has remarkable hazardous effects on both the environment and human health, as it is known to block the iodine uptake by the thyroid gland.[1,2] The accurate determination of SCN^- levels in marine water can also be of paramount importance for the detection of live coral reef fishes collected using an illegal and highly destructive technique—cyanide fishing.[3,4] Live fishes traded for human consumption or marine aquariums which have been exposed to cyanide are known to naturally excrete SCN^- as a way of self-depuration.[5] If SCN^- levels recorded in the water holding recently imported fishes exceed the basal values recorded for seawater, this can be considered as evidence that imported fishes were actively excreting SCN^- and have been illegally collected with cyanide.

Several analytical methods for the determination of SCN^- in various types of samples have been previously reported elsewhere, with molecular spectroscopic methods (including UV-Vis spectrophotometry and spectrofluorimetry) being the most widely applied.[1,6,7] The most used spectrophotometric method for thiocyanate determination is based on the formation of a red complex ($\lambda = 480$ nm) with ferric ion, $Fe(SCN)^{2+}$, in acid medium.[1]

Other methods used for determination of SCN^- are electrochemical methods (with ion selective electrodes),[8] gas chromatography with electron capture,[9] capillary electrophoresis,[10] sensors based on fluorescence detection,[11] and liquid chromatography coupled to an UV detector (LC-UV).[12] Most of these methods either require sophisticated operation or take long operation times. Additionally, all the methods referred, with exception of LC-UV, cannot be directly applied to the determination of thiocyanate in seawater. Therefore, there is still need for the development of a simple, fast and reliable method for determination of SCN^- in seawater.

Optical fiber (OF) sensors and OF based methodologies have been proven to be of high sensitivity and of easy operation for several analytes.[13–26] In OF sensors based on polymeric film as

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Environmental impact

Thiocyanate (SCN^-) has remarkable hazardous effects on both the environment and human health, as it is known to block the iodine uptake by the thyroid gland. Fishes exposed to cyanide, a highly destructive technique used for coral reef's fish collection, are known to naturally excrete thiocyanate as a way of self-depuration; these organisms are traded live and present a high market value both for human consumption and/or the marine aquarium trade. Therefore, obtaining reliable data on SCN^- concentration in seawater becomes an important aim both in environmental sciences and sensing technology. An analytical methodology based on optical fiber detection has been developed for thiocyanate assessment in seawater. This methodology is capable of inexpensive, fast and direct analysis of thiocyanate in seawater samples and therefore could be applied to non-invasive and non-destructive detection of coral reef fishes collected using cyanide fishing.

the sensitive material/component, the analytical signal generation and characteristics must be understood as an interaction between several physical and chemical factors. Firstly, the analytical signal depends on the wave guide, the polymeric cladding and the analyte properties. Secondly, it is important to take into account the molecular interactions that occur between the analyte molecules and the polymeric film with consequent changes in the intensity of the light power guided through the OF.[27,28] When the analyte molecules contact with the polymeric film, the optical power intensity will vary proportionally to the amount of the analyte present. Without losing sight of the advantages of OF sensors, the aim of this work was to develop an OF based methodology for detection of thiocyanate in seawater samples. The developed methodology was validated by comparison with a high performance liquid chromatography with UV detector (HPLC-UV) methodology and applied to the determination of thiocyanate in seawater samples.

Experimental

Preparation of calibrants for thiocyanate determination

Standard solutions of SCN^- (4, 50, 100, 200, 300 and 400 μg L^{-1}) have been prepared by dilution of NaSCN, (Sigma, Sintra, Portugal) in artificial seawater. Artificial seawater was prepared using freshwater purified by a reverse osmosis unit and mixed with the synthetic salt Crystal Sea® produced by Marine Enterprises International® (Baltimore, MD, USA), following the instructions of the manufacturer.

Preparation of standard solutions for comparison of methodologies

In order to test and compare the performance of the proposed LC-OF methodology with HPLC-UV, ten different concentrations of SCN^- (10, 70, 100, 130, 190, 220, 250, 310 and 370 μg L^{-1}) have been prepared, and determined with both methodologies performing five repeated evaluations for each concentration tested.

OF based methodology

The schematic representation of the experimental apparatus of OF based methodology is shown in Fig. 1a. The injector unit (I) was connected to the pump (P) which was connected to the mobile phase flask (MP). The column (C) (150 mm × 4.6 mm i.d., C30 column modified with 5% polyethylene glycol (PEG) 20 000) was connected to the injector unit (I) and to the analytical tube (AT). The AT had an internal narrowed region of 0.4 cm diameter and 6.5 cm long having inside the OF that is a monomode optical fiber pigtail, core and cladding diameters of 9 and 125 μm, respectively, integrated into a directional 50:50 Y optical coupler (OC), with an angle FC/PC connector and a super FC/PC on the input ends. The optical fiber was previously uncladded, cleaved to 15 mm of the optical path cord and dipped (the cleaved section) into a PEG solution, by dip-coating technique, resulting in the sensitive component of the OF detection. Tests concerning the stability/durability of the sensitive OF section by optical signal monitoring during 3 months and SEM observation of the sensitized OF section revealed that no significant signal degradation and morphology changes were recorded, respectively. Additionally, the sensitive component (uncladded OF section + polymeric film) of the OF methodology can be replaced, after 3 months of continuous operation, in order to maintain high analytical performance after extensive utilization for longer periods of the analytical sensing system. Besides the coated optical fiber and the analytical tube, the detection component of the OF based methodology was constituted by a laser diode optical source (L, Oz Optics, Ottawa, Canada) and a photodiode detector (PD, Oz Optics, Ottawa, Canada). The laser diode (1 mW) optical source was (a) set at 1550 nm for working wavelength and at continuous waveform (CW) regarding the operational mode frequency, to generate the interrogating signal, and (b) connected to the optical coupler (OC). The OC was also connected to the photodiode detector (P), which measures the intensity of the modulated signal, and to a laptop (PC) with homemade software.

Standards of SCN^- (4, 50, 100, 200, 300 and 400 μg L^{-1}) and seawater samples (20 μL) were introduced by a micro-syringe (Hamilton, Bonaduz, GR, Switzerland) at the top of the injector (I). After separation on the column the analytes reached the analytical tube (AT), which contains the coated OF, generating an analytical signal (Fig. 1b). The changes in the reflected optical power caused by variations in the OF refractive index have been previously discussed by Silva *et al.*,[13–23] and depend on the coating film (PEG) and analyte properties, as well as the chemical interactions which can take place between these two elements. The mobile phase was 300 mM sodium sulfate and 50 mM sodium chloride and the flow-rate was 1.0 mL min^{-1}.

Calibration models were built by injection of 20 μL of different concentrations (4, 50, 100, 200, 300 and 400 μg L^{-1}) of standard solutions. The concentration of thiocyanate was determined by direct interpolation in the calibration curve within their linear dynamic range, and the detection limits were calculated using $y = y_B + 3s_B$, where s_B is the standard deviation (SD) of the blank signal estimated as $s_{y/x}$, the residual SD taken from the calibration line, and y_B is the blank signal estimated from the intercept taken also from the calibration line.[29]

HPLC-UV based methodology

An HPLC-UV based methodology[12] was used in order to test and compare the performance of the OF methodology. The column used was C30 column (150 mm × 4.6 mm i.d.) modified with 5% PEG 20 000. The mobile phase was 300 mM sodium sulfate and 50 mM sodium chloride and the flow-rate was 1.0 mL min^{-1}. Standards of thiocyanate were injected (20 μL) on a HPLC-UV (Jasco PU980, Easton, USA) and the wavelength of UV detection was 220 nm.

Sampling of thiocyanate

Seawater samples were collected in clear screw neck glass vials (Fisherbrand, Fisher Scientific, UK) during the months of June and July 2010 from several locations off Aveiro in the Portuguese Coast (Fig. 2). Collected samples were filtered through a 0.45 mm membrane filter and kept under refrigeration at 4 °C before further analytical processing.

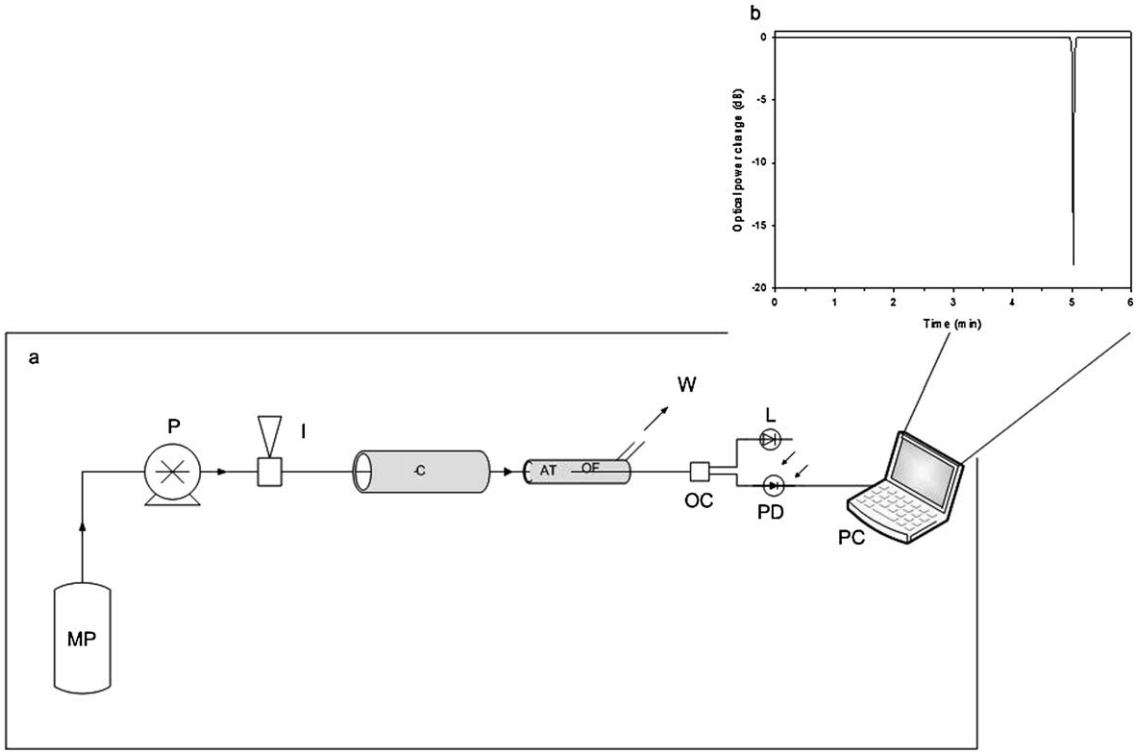

Fig. 1 (a) Schematic representation of the experimental apparatus used for OF based methodology (MP = mobile phase flask; P = pump; I = injector; C = column; AT = analytical tube; OF = optical fiber; W = waste; OC = optical coupler; L = laser diode optical source; PD = photodiode detector; PC = laptop with homemade software); (b) analytical signal obtained by OF based methodology for a standard of thiocyanate.

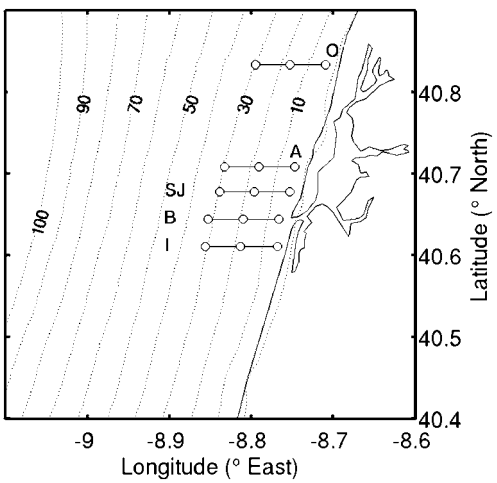

Fig. 2 Study area and sampling locations off Aveiro in the Portuguese Coast. A—Aveiro, B—Barra, I—Ilhavo, O—Ovar and SJ—São Jacinto.

Results and discussion

Table 1 summarizes the main analytical characteristics obtained for thiocyanate with the two tested methodologies. The analytical time (less than 6 min) was the same for both approaches, since the same chromatographic components and operational conditions (such as the mobile phase) were used.

In terms of retention time, linear range and detection limit, the results achieved were in the same order of magnitude for HPLC-UV and OF based methodologies. Both approaches displayed a retention time of approximately 5 min, a linear range from 4 to 400 μg L^{-1}, while the detection limits were 4 μg $L^{-1}$and 3 μg L^{-1} for the HPLC-UV and OF based methodologies, respectively.

From the calibration study, it was also found that the analytical performance of the OF based methodology reaches a plateau in terms of maximum capacity of detection for concentration values much over the linear range,[30] particularly for concentrations higher than 500 μg L^{-1}.

Ten standards with different concentrations of thiocyanate were used to evaluate the performance of the OF methodology *versus* the HPLC-UV methodology. The statistical analysis[31] of the obtained results suggests: (a) that a linear correlation can be established between the two analytical methodologies for the analysis of thiocyanate, with squared correlation coefficient R^2 of 1 ($p < 1.59 \times 10^{-33}$); (b) that the results obtained with the two analytical methodologies cannot be statistically differentiated since the regression line has an intercept and a slope not significantly different from 0 ($p = 0.944$) and 1 ($p < 1.59 \times 10^{-33}$), respectively; and finally (c) that there is a low dispersion level of the results obtained by the two applied analytical methodologies for thiocyanate analysis supported by an extremely narrow interval at a 95% confidence level.

The error, that is ((found value − expected value)/expected value) × 100) was lower than 0.4% for the OF methodology, and lower than 0.3% for the HPLC-UV methodology.

Apart from keeping the quality of the analysis of SCN^- at the same pattern of other usual methods of analysis, *i.e.* HPLC-UV, the developed methodology compares advantageously due to: (a)

Table 1 Analytical parameters obtained for thiocyanate with HPLC-UV and OF based methodologies

	HPLC-UV	OF
Retention time/min	5.14	5.02
Linear range/μg L^{-1}	4–400	4–400
Linear calibration	$y = 3.52 + 0.147x$	$y = 2.77 + 0.073x$
Squared coefficient of correlation/R^2	0.9999 ($p < 6.33 \times 10^{-9}$)	0.9999 ($p < 3.57 \times 10^{-9}$)
Detection limit/μg L^{-1}	4	3

easiness of operation and effective cost of equipment, (b) compact and versatile design of the analytical system, (c) possibility of remote data acquisition, and (d) high potential for miniaturization, allowing the possibility of *in situ* analysis.

The compact design (Fig. 1) of the OF based methodology allows the integration of the system components into a portable structure for field application, *i.e.*, for thiocyanate *in situ* analysis. This structure should include a sampling tube and a mini-valve, automatically controlled by software and connected to the pump (P, Fig. 1). An electronic platform/software should be also implemented in order to (a) provide direct readings of the analytical signal or (b) database for measurements storage, and (c) remote data acquisition by integration in a wireless network.

After development, comparison and validation against HPLC-UV methodology, the OF methodology was applied to the analysis of thiocyanate in 15 seawater samples collected off Aveiro in the Portuguese Coast. The results from this analysis are summarized in Table 2.

In Table 2, it can be observed that the higher values of thiocyanate were found near the coast (1 km) decreasing to below the detection limit with increasing the distance to the coast. This fact could be related to anthropogenic activities since at off-shore there are larger water bodies which decrease this effect resulting in a decrease of SCN^- levels.

Moreover, the levels of thiocyanate found in the water samples collected on the Portuguese coast were in the same range of levels found for seawater samples collected on the Japanese coast (8.1–15.0 μg L^{-1}) by Rong *et al.*[12]

Table 2 Concentration of thiocyanate in seawater samples collected off Aveiro in the Portuguese Coast (mean and standard deviation obtained for five experiments)

Sampling site	Distance to the Coast/km	Thiocyanate/μg L^{-1}
Aveiro	2	20.5 ± 0.3
Aveiro	17	<3.3
Aveiro	24	<3.3
Barra	1	21.3 ± 0.3
Barra	5	7.2 ± 0.1
Barra	8	<3.3
Ilhavo	1	22.7 ± 0.3
Ilhavo	5	<3.3
Ilhavo	9	<3.3
Ovar	1	18.2 ± 0.2
Ovar	7	<3.3
Ovar	26	<3.3
São Jacinto	1	16.0 ± 0.2
São Jacinto	4	5.1 ± 0.1
São Jacinto	8	<3.3

Taking into account the complexity of seawater, and in order to access the applicability of the OF based methodology for the analysis of SCN^- in this matrix, the potential interference of iodide (concentration of 90 μg L^{-1}) in thiocyanate detection was evaluated. No interference could be detected, as iodide produces a peak in a different time window (around 2 min) of the target analyte (SCN^-), as shown in Fig. 3. Moreover, interferences from other analytes such as iodate were also evaluated and no interference was observable since the peak produced was also in a different time window (at 1.20 min).

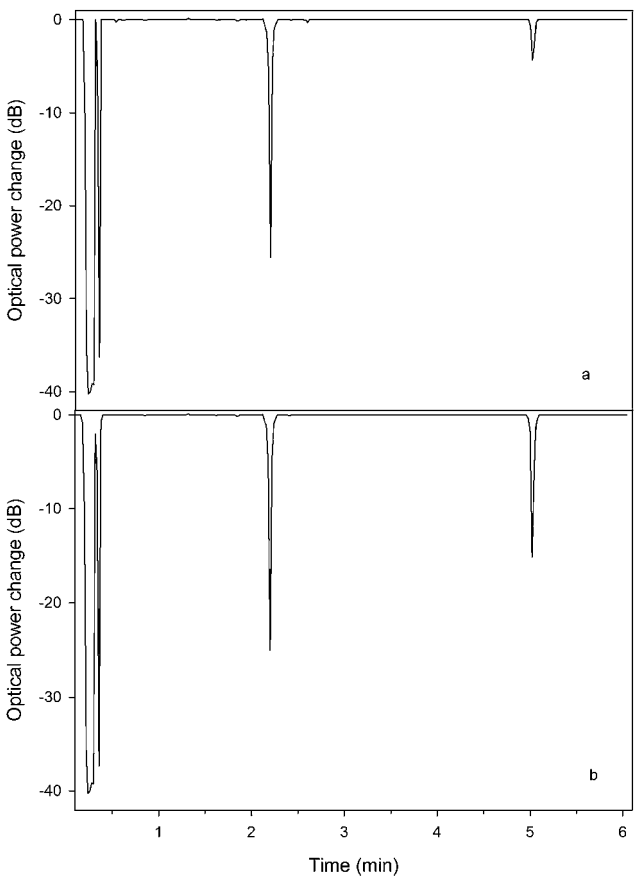

Fig. 3 Analytical signal obtained by OF based methodology (a) for a sample of seawater from Barra (off Aveiro in the Portuguese Coast) with a distance to the coast of 1 km, and (b) for a sample of seawater from Barra with a distance to coast of 1 km with thiocyanate standard addition. Iodine is the peak at 2.20 min and thiocyanate is at 5.02 min.

Conclusions

The OF methodology showed a high analytical performance, mainly in terms of linear range (4–400 μg L^{-1}) and detection limits (3 μg L^{-1}). It has proven to be adequate for the assessment of thiocyanate in seawater and comparable to HPLC-UV methodology. Simple and fast analysis of thiocyanate in seawater, easiness of use and compact design were also analytical features of the developed OF based methodology. In this way, this new methodology can constitute a platform for inexpensive analysis of thiocyanate in environmental samples without iodide interferences. A good example for a practical application of this new methodology is the non-invasive and non-destructive detection of highly priced coral reef fishes collected with cyanide (by rapidly and accurately tracing abnormally high levels of SCN^- in the water holding these organisms, rather than sacrificing fish to detect cyanide levels in their tissues and/or blood).

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